High genetic diversity including potential new subtypes of hepatitis C virus genotype 6 in Lao People's Democratic Republic

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Abstract

Sera from 105 anti-HCV-positive first-time blood donors collected in 2004, 2005 and 2008 in different provinces in Laos were investigated by PCR. Forty-five samples were positive for HCV (42.86%); two belonged to subtype 1b (2/45, 4.4%) and all others to genotype 6 (43/45, 95.6%), including subtypes 6b, 6h, 6k, 6l, 6n and 6q. Three groups of sequences were not clearly attributable to any genotype 6 subtype, two of which may be regarded as candidates for new subtypes of genotype 6. Two samples were mixed infected with different subtypes or clusters of genotype 6 viruses.

Keywords: Genotype, HCV, Laos, phylogeny, subtype
Original Submission: 20 April 2011; **Revised Submission:** 18 August 2011; **Accepted:** 30 August 2011
Editor: G. Antonelli
Article published online: 2 September 2011

Clin Microbiol Infect 2011; **17:** E30–E34
10.1111/j.1469-0691.2011.03665.x

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Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma worldwide. It is estimated that up to 3% of the world's population are infected with HCV [1]. Currently, six genotypes of HCV are recognized [2] and a seventh has been proposed [3]. While some of the genotypes, such as 1, 2 and 3, are found worldwide, others show a geographically more restricted prevalence [4–6]. Genotype 6, which is found throughout East Asia, is by far the most variable genotype, with at least 23 proposed subtypes (a–w) [2,4,5,7–12]. While HCV genotypes have been well studied in some East Asian countries such as China [13–15] or Thailand [7,16,17], only a few strains have been reported from other countries [5].

Forty-eight, 52 and five serum samples were collected from anti-HCV-positive first-time blood donors in 2004, 2005 and 2008, respectively. Seventy-eight sera were from men, 27 from women. The majority of the donors were residents of Vientiane city (n = 73), the others lived in Vientiane province (n = 14), Borikhamxay province (n = 13) and in Luang Prabang province (n = 5). The age of the donors ranged from 17 to 57 years (mean, 30.2).

Amplification of the HCV core/E1 gene region was done as described before [18], with the exception that 1 μL of cDNA was used for the first-round PCR and 1 μL of first-round product for the semi-nested PCR. The NS5B region was amplified according to Sandres-Saune *et al.* [19], but using an annealing temperature of 60°C for all cycles. The primers used to amplify the core/E1 region were used previously to amplify 1a, 1b, 3a, 4a and 4d sequences [18] and the NS5B primers previously amplified 1a, 1b, 1c, 2a, 2b, 2c, 2k, 3a, 4a, 4d, 4f, 4i and 5a [19]. Samples suspected to be mixed infected were cloned using the pCR4-TOPO kit (Invitrogen, Merelbeke, Belgium). Phylogenetic analysis was performed using MEGA4 [20] and GENEIOUS PRO5.3.4 (Biomatters Ltd, Auckland, New Zealand). Distance calculations were also done with MEGA4.

Of the 105 anti-HCV-positive donors 35 and 44 were RNA positive using the core/E1 and the NS5B gene PCRs, respectively. Thirty-five and 40 fragments suitable for phylogenetic analysis were obtained from these regions. From 30 samples, sequences of both regions were available (GenBank accession numbers for all sequences: HE580284–HE580419).

Phylogenetic analysis of the 865 nt region of the core/E1 gene showed that two samples (47054 and the clones of 47015) belonged to genotype 1, subtype 1b, and that all others belonged to genotype 6 (Fig. 1a). One sample each was attributed to subtypes 6h (47052), 6n (47069), 6l (47013) and 6k (one set of clones from 47098), two samples to 6q (47087, 47036) and three to 6b (47011, 47017 and the clones of 47049) (Fig. 1a). The other sequences could not be clearly

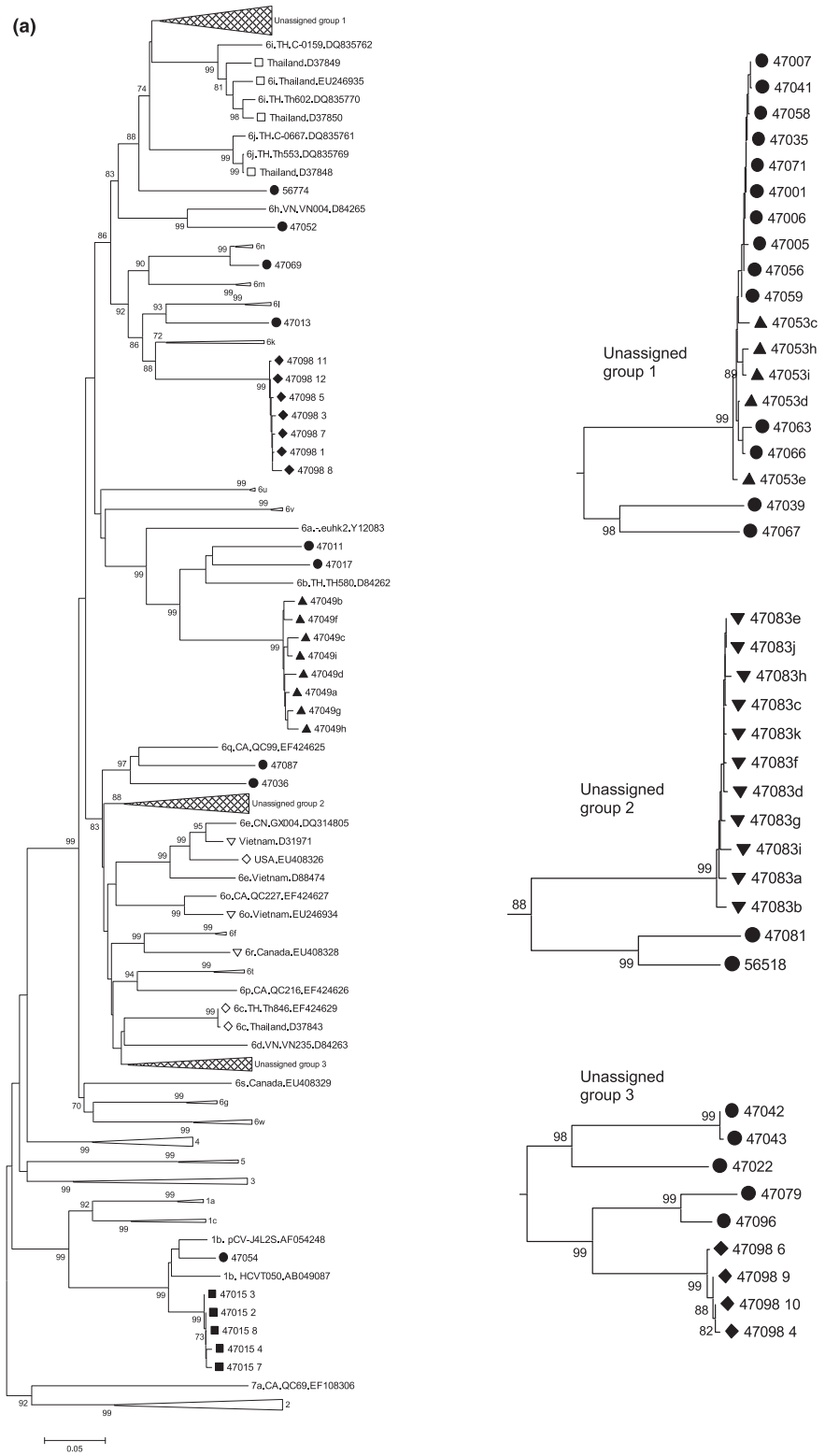

FIG. 1. Phylogenetic tree based on (a) 865 nt of the HCV core/E1 gene region and (b) 340 nt of the NS5B region. Neighbour-joining and Kimura two parameter methods were used and only bootstrap values ≥70 are shown. The clustering was largely confirmed by different phylogenetic methods, except that the two clusters of unassigned group 3 were sometimes separated. Sequences obtained during the present study are marked in black (dots are used for original sequences; diamonds, triangles and squares for clones), and the closest BLAST fits are shown as open symbols (squares are from sequences in unassigned group 1, triangles from sequences in unassigned group 2 and diamonds from sequences in unassigned group 3).

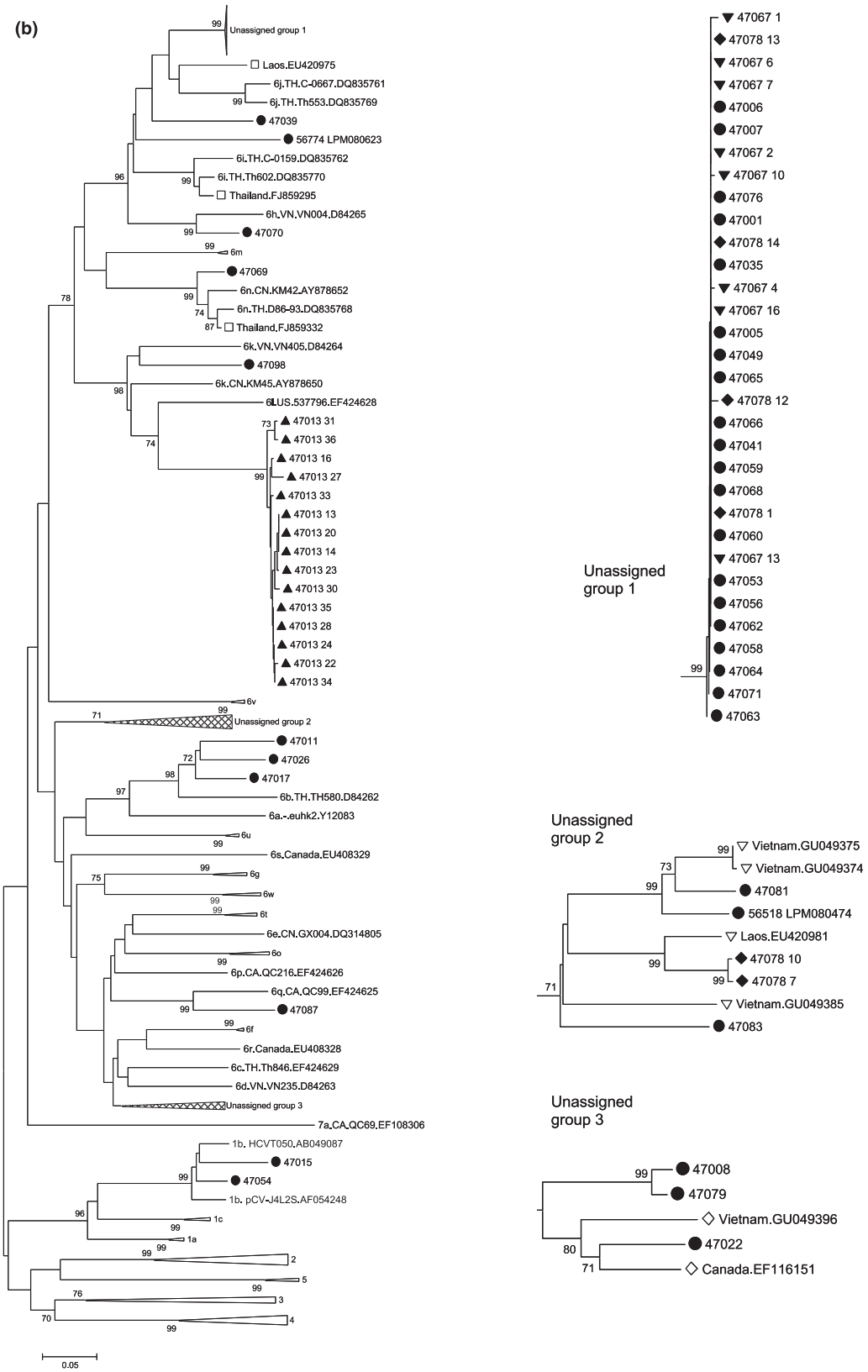

FIG. 1. (Continued)

assigned to a genotype 6 subtype based on this analysis (Fig. 1a, unassigned groups 1–3). Of the five samples suspected to be mixed infected based on the original sequence data (47049, 47053, 47098, 47083 and 47015), only one (47098) was confirmed by the different clustering of the clones: one set with subtype 6k, the other set in unassigned group 3 (Fig. 1a).

Phylogenetic analysis of 340 nt of the NS5B region assigned two strains (47015 and 47054) to 1b and all others to genotype 6 (Fig. 1b). One strain each belonged to 6h (47070), 6n (47069), 6k (47098), 6l (clones of 47013) and 6q (47087) and three sequences to 6b (47011, 47026, 47017). The other 30 strains clustered inconclusively (Fig. 1b, unassigned groups 1–3). Of the three suspected mixed infected samples (47013, 47067 and 47078), only the clones of 47078 clustered differently (with the first and second set of unassigned sequences, Fig. 1b), confirming the mixed infection.

There was a good overall agreement between the results obtained for the two different genomic regions with only two sequences showing a distinct clustering (47049, core/E1, 6b; NS5B, unassigned group 1; 47067, core/E1, second cluster of the first unassigned group; NS5B, first cluster of the first unassigned group) (Fig. 1).

Genotype 6 of HCV is largely predominant in Laos (43/45, 95.6%), while only two sequences of the worldwide prevalent subtype 1b were found and no subtype 3a, which was suggested to be the most common subtype in south-east Asia [7]. Thus, the genotype prevalence in Laos is quite distinct from that in the surrounding countries, which may be due to less exposure to public health mass interventions [5]. The six genotype 6 subtypes detected in the present study were reported previously mainly from south-east Asia and none of them seems to be restricted to Laos [5,7]. Unassigned group 1 seems to represent a distinct cluster of 6j or 6i, due to its clustering in the phylogenetic trees of Fig. 1 and its minimal genetic distance of <15% to subtype 6j (Table 1). More sequences similar to outlier 56774 are needed before the status of this distinct strain can be resolved. Groups 2 and 3 may be considered as candidates for new subtypes of genotype 6. Both have high genetic distances (at least 18.5%) to the next closest subtypes (Table 1) and each group contains sequences from several individuals from different non-contiguous regions in Laos (group 2, donors from two different districts of Vientiane city and from Luang Prabang (56518); group 3, donors from three different districts of Vientiane city and from Borikhamxay province (47098)) and even sequences from different countries (group 2, Laos and Vietnam; group 3, Laos, Vietnam and Canada). The high maximal genetic distance between the Laos strains within the two groups (at least 21.3%) and the interspersed clustering with sequences from the other countries, as well as circumstantial evidence, virtually exclude a direct transmission between the donors. No amino acid mutations specific for and exclusively found in the Laos strains of unassigned groups 2 and 3 were found. Thus the designation as a new subtype would be of epidemiological value according to the criteria proposed by Simmonds *et al.* [2]. As the two clusters of group 3 differ by at least 19% from each other and by at least 18.3% from the next closest subtypes (Table 1), it is conceivable that group 3 comprises two new potential subtypes.

TABLE 1. Minimal genetic distances of unassigned groups and outliers to the next closest subtypes for the core/E1 and the NS5B regions. For comparison, the minimal genetic distance between the reference strains of the two most closely related genotype 6 subtypes is given

	Minimal genetic distance and closest subtype (core/E1)	Minimal genetic distance and closest subtype (NS5B)
Unassigned group 1	17.7% (6j)	14.6% (6j)
Outlier 56774	18.8% (6j)	22.5% (6i)
Outlier 47039	NA	16.9% (6i)
Unassigned group 2	20.8% (6o)	28.9% (6d)
Unassigned group 3	18.5% (6c)	20.7% (6c)
Group 3, cluster 1	19.0% (6c)	19.9% (6d)
Group 3, cluster 2	18.3% (6c)	20.6% (6c)
Genotype 6 reference strains	13.9% (6f and 6r)	17.6% (6f and 6r)

In the present study, less than half of the samples from anti-HCV-positive blood donors were also PCR positive (45/105, 42.86%). In some donors this may result from spontaneously controlled HCV replication after acute infection [1], but may in part also be due to suboptimal storage.

Transparency Declaration

The present study was financially supported by the Ministry of Cooperation of the Grand-Duchy of Luxembourg and by the Centre de Recherche Public—Santé, Luxembourg. The authors declare that there is no conflict of interest related to the present manuscript.

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